Genotypic Detection of Polyhydroxyalkanoate-producing Bacilli and Characterization of *pha*C Synthase of *Bacillus* sp. SW1-2

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Abstract: A group of bacilli phenotypically screened for synthesis and intracellular accumulation of PHAs granules by the use of Sudan Black B stain, eight strains were detected. Pair of specific PCR primers was designed and applied for genotypic detection of *pha*C synthase gene. Approximately, 760 bp DNA fragment was successfully amplified in the eight strains. Among the positive strains, *Bacillus* sp SW1-2, produced 36 g/L of the biopolymer during growth on modified E2 medium supplemented with glucose. Spectroscopic analysis by C^{13}NMR and H^{1}NMR revealed four narrow peaks (lines) (CH3; 21.2 ppm, CH2; 42.7, CH; 68.5 and C=O; 169.7 ppm) and 3 groups of signals (2.45, 2.58 and 5.2 ppm) identical and characteristic to polyhydroxybutyrate (PHB); respectively. Furthermore, the amplied PCR fragment, from genomic DNA of *Bacillus* sp SW1-2, was cloned in pGEM-T-Easy vector and sequenced with universal T7 and SP6 primers. The sequence showed 99% identity to *pha*C gene for polyhydroxyalkanoate synthase of many *B. megaterium* strains deposited in Genbank. While, showed 73% and 72% identity to synthases of *Bacillus mycoides* and *Bacillus* sp. INT005, respectively.
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1. Introduction

Polyhydroxyalkanoates (PHAs) are group of polyesters synthesized and intracellular accumulated by several microorganisms, especially when the carbon substrate is in excess to other nutrients (Sanger et la., 1977; Madison and Huisman, 1999; Kimm and Lenz, 2001; Steinbuchel, 2001; Shamala et al., 2003; Reddy et al., 2003). PHAs can be classified into different types according to the number of repeating units in the polymers. These polyesters are biodegradable and biocompatible thermoplastics (Madison and Huisman, 1999; Ojumu et al., 2004). Among PHAs, polyhydroxybutyrate (PHB) is the best known polyester due to its similarity to synthetic petroleum–based plastics (Mokhtari-Hosseini et al., 2009). PHB has many applications in medicine, veterinary practice, tissue engineering materials, food packaging and agriculture (van der Walle et al., 2001; Zinn et al., 2001; Borah et al., 2002; Luengo et al., 2003; Chen and Wu, 2005).

Currently, there are variety of methods for selecting polyhydroxyalkanoate (PHA)-accumulating bacteria. These include both phenotypic and genotypic detection methods. The first apply stains; Sudan Black staining (Schlegel et al., 1970), Nile blue A staining and direct staining of bacterial colonies by fluorescence (Spiekermann et al., 1999). While, genotypic detection methods based on the use of various polymerase chain reaction (PCR) protocols (Sheu et al., 2000; Solaiman et al., 2000; Shamala et al., 2003; Solaiman and Ashby, 2005). Indeed, genotypic methods have become a highly sensitive and precise tool for detecting and amplifying the gene encoding PHA synthase.

Among PHB producer, members belong to genus *Bacillus* have been reported and extensively studied (Chen et al., 1991; Caballero et al., 1995; Labuzek and Radecka, 2001; Wu et al., 2001; Belam et al., 2002; Borah et al., 2002; Satoh et al., 2002; Tajima et al., 2003; Valappil et al., 2007b; Valappil et al., 2008; Adwitiya et al., 2009; Reddy and mahmood, 2009; Gurunathan et al., 2010). Recently, molecular investigation on polymer production revealed that poly (3-hydroxybutyrate) biosynthesis genes are *phb*A, *phb*B, and *phb*C (PHB synthase); these genes have been cloned and expressed in *E. coli* and their genetic organization has been extensively reported (Madison and Huisman, 1999; Zhang et al., 2001; Enan and Bashandy, 2004). PHA synthases (*pha*C) are key enzymes in PHA producing polymer granules. These enzymes are classified into four classes according to genetic sequence- deduced primary structure, substrate specificity and subunit composition (Steinbuchel, 2001). Novel PHA synthase from *Bacillus megaterium* required PhaC and PhaR for activity in vivo and in vitro reported by (McCool and Cannon, 2001). PhaCBm showed greatest similarity to the PhaCs of class III in both size and sequence. Unlike those in class III, the 40-

kDa PhaE was not required and the 22-kDa PhaRBm protein had no obvious homology to PhaE.

The main objective of this study was the phenotypic as well as genotypic detection of PHAs production and intracellular accumulation by a group of bacilli isolated from soil and sewage samples. Production of PHB biopolymer from *B. megaterium* SW1-2 and chemical characterization of the produced polymer by C^{13}NMR and H^{1}NMR spectroscopy was closely investigated. Special emphasis was given to molecular characterization of *pha*C synthase of *B. megaterium* SW1-2 by use of specific primers. Degree of identity of the nucleotide sequence compared to corresponding sequences of *pha*C synthases of other bacilli was also reported.

2. Materials and Methods

Microorganism; enrichment and isolation

Screening was carried out by enrichment and isolation of spore-forming bacilli from sewage and soil samples obtained from Dammam, Eastern province, Saudi Arabia. Samples were treated for 20 min at 90 ^{o}C and subsequently cultivated on nutrient agar medium. Purified strains were cultived on modified E2 medium supplemented with glucose as carbon source. Screening for PHB accumulation was carried out by staining with Sudan Black (0.3% (w/v) in 70% ethanol). Bluish-black colonies indicating PHB production and thus used for further studies (Belma et al., 2002). Isolates were maintained on nutrient agar slant composed of (g/L): peptone; 5, beef extract; 3, NaCl; 2 and agar; 20. Stock cultures were subcultured at regular intervals of one month and stored under refrigeration. Potential PHB producing strain, *Bacillus* sp SW1-2, was molecularly identified by 16S rDNA analysis as previuosly described (Berekaa and Al Thawadi, 2012).

Growth and biopolymer production

The bacterium was grown in 50ml aliquot of nutrient broth dispensed in 250ml Erlenmeyer flask and incubated at 37°C for 24h at 150 rpm. 1.5% inoculums of the overnight culture was used to inoculate modified E2 medium of the following composition (g/L): ammonium sulfate; 2.5, glucose; 20, KH_2PO_4; 1.5, Na_2HPO_4; 3.5, $MgSO_4.7H_2O$; 0.2, beef extract; 0.5 and trace element solution composed of (g/L); H_3BO_3, 0.3; $NaMoO_4.2H_2O$, 0.03; $CuSO_4.5H_2O$; 0.01, $CuSO4.5H_2O$; 0.01; $NiCl_2.6H2O$, 0.02; $ZnSO_4.7H_2O$, 0.1 and traces of ammonium ferric citrate. 50 ml of the medium placed in 250 ml Erlenmeyer flasks were inoculated with 750 µl of the pre-culture. At the end of incubation period, PHB was determined and the cell dry weight was estimated.

Extraction of PHB from *B. megaterium* SW1-2

PHB was extracted from the cell masses by using modified Hypochlorite method (Rawte and mavinkurve, 2002). For this purpose, *B. megaterium* cells were grown in 250 ml Erlenmeyer flasks containing 50 ml of the modified E2 medium. At the end of incubation period, one ml of cell suspension was centrifuged at 6,000 rpm for 15 min. Cell pellet was washed once with 1 ml saline and was re-centrifuged. Subsequently suspended in equal volume of sodium hypochlorite (5.5% active chlorine) and incubated at 45^{o}C for 60 min. This extract was centrifuged at 10,000 rpm for 20 min and the pellet of PHB was washed with water and twice with ethanol:acetone mixture (2:1). The pellet was again centrifuged at 10,000 rpm to get purified PHB. Determination of PHB yield was performed routinely by dry weight estimation. The ultraviolet (UV) absorption spectrum of the polymer was analyzed following its conversion to crotonic acid by treatment with concentrated H_2SO_4, and the absorbence was scanned between 200 and 350 nm with UV-1800 spectrophotometer (Shimadzu Scientific, USA). For dry weight estimation, the pellet after extraction was dried to constant weight.

Analysis of biopolymer

Cell dry weight (CDW)

After centrifugation of the culture medium, the supernatant was discarded and the cell pellet was washed with distilled water. The washed pellet was resuspended in 1 ml distilled water, transferred to pre weighed boats and dried to constant weight at 60^{o}C.

Characterization of extracted PHB by C^{13}NMR and H^{1}NMR analysis

Extracted PHB biopolymer of *B. megaterium* SW1-2 was characterized by spectroscopy analysis. H^{1} NMR spectrum was recorded on a JEOL JNM-LA 500 MHz spectrometer 30^{o}C in $CDCl_3$ as solvent. While, C^{13}NMR spectral experiments were performed at 125.65 MHz with the following acquisition parameters: 32 k data point, 0.967 s acquisition time, recycle delay 1 s and contact time 4.50 ms.

Bacterial strains and plasmids

Nucleic acid techniques

Genomic DNA was isolated by the use of DNA isolation kit (QIAamp DNA Mini, QIAGEN) according to the manufacturer instructions, with some modification. Lysozyme (20mg/ml) in TE buffer was used for lysis of the cells by incubation at 37^{o}C for 30 min. Recombinant *E. coli* DH5a cells harboring recombinant plasmids were screened separately by blue-white selection utilizing LB-agar plates supplemented with ampicillin and IPTG.

PCR Technique

PCR reaction was carried out in a final volume of 50 ul using HotStar PCR Master Mix Kit,

according to the manufacturer instructions (QIAGEN). The PCR reaction contained 1 ul of each primer (10 pmol). The PCR condition was 1 cycle at 95°C for 15 min followed by 35 cycles at 95°C for 55 sec, 58 °C for 55 sec, and 70°C for 55 sec. The final extension step was carried out at 72°C for 10 min and PCR reaction was run in Eppendorf Mastercycler personal, Germany.

Designing specific primers

Pair of specific primer, corresponding to two conserved regions (Figure 1), was designed for amplifying Class IV *pha*C1 gene by aligning and analyzing Class IV PHA biosynthesis operon sequences belonging to the following *Bacillus* species: *Bacillus megaterium*1 AB525783, *Bacillus* sp. INT005 AB077026, *Bacillus megaterium*2 AF109909, *Bacillus* sp. CFR13 HM370560, *Bacillus megaterium*3 strain BPK-3 GU190757, *Bacillus cereus* strain DC4 HM122247, *Bacillus cereus* strain DC3 HM122246, *Bacillus cereus* strain DC2 HM122248. The primers designs were; forward primer P1:5'-GAT GTG TAT TTG CTT GAC TGG GG-3', reverse primer P2: 5'-AGC CAA TCG CCG ATT GAA GGA TA-3'). Primers were synthesized by *metabion*, Martinsried, Germany.

Detecting PCR products

Electrophoresis on 1% (w/v) agarose gels (1X TAE) was used for detecting PCR amplification products. Λ DNA/*Hind* III (0.2 µg/µl) was used as DNA size marker. Run conditions were 6.5 volts/cm for 2 and half hrs. The gel was stained with a 0.5 µg/ml ethidium bromide solution and amplified DNA fragments were visualized under UV light and recorded with a GelDoc image digitalizer (Bio-Rad).

DNA sequencing data analysis

Analysis of nucleotide sequences was performed using BioEdit computer based program. Alignments and comparison of sequences were carried out using Blast program. The nucleotide sequencing data reported in this work submitted to the GenBank nucleotide sequence database and is listed under the accession number JQ755810.

Cloning and Sequencing of the PCR Products

To ligate the generated PCR products onto pGEM-T-Easy vector (Promega Co.), 2 ul was taken in a clean 0.5 ml tube to which 1 ul pGEM-T-Easy vector (50 ng) and 1 ul ligase buffer were added, followed by the addition of 2 U ligase enzyme. Final volume of the ligation reaction was adjusted to 10 ul by the addition of nuclease-free distilled water. The tube was incubated at 16°C for 16 h. Transformation of *Escherichia coli* DH5a competent cells was carried out according to Sambrook et al. (1989). Recombinant *E. coli* DH5a harboring the pGEM-T-Easy vector was screened in selective LB/IPTG/X-gal/Ampicillin/agar plates. Plasmids were prepared from some positive clones using the PureYield Plasmid Miniprep System (A1222, Promega, USA). Sequencing of the PCR products cloned onto pGEM-T-Easy vector (four different clones) was carried out according to Sanger et al. (1977), using the MegaBACE 1000 DNA Sequencing System (Pharmacia/Amersham Co.). The chain termination sequencing reaction was conducted utilizing the DYEnamic ET terminator kit as an integral part of the MegaBACE 1000 DNA sequencing system. Sequencing reaction products were purified using DyeEx 2.0 Spin Kit (63206 QIAGen) and applied to MegaBace 1000 Sequencing machine.

3. Results and Discussion

Enrichment and isolation of PHAs accumulating bacilli

A group of endospore-formers bacilli was subjected to phenotypic and genotypic detection of PHAs synthesis. Bacilli were selectively enriched and isolated from sewage and soil samples from Dammam, Saudi Arabia, by heat treatment and subculture on nutrient agar medium. Purified strains were phenotypically screened for PHAs synthesis and intracellular accumulation by cultivation on modified E2 medium supplemented with glucose as a sole carbon source. Eight strains revealed purple-blue colored colonies after stain with Sudan Black B. In accordance, Sudan Black staining (Schlegel et al., 1970), Nile blue A staining and direct staining of bacterial colonies by fluorescence (Spiekermann et al., 1999) were used as phenotypic methods of detection. For genotypic detection of *pha*C synthase gene, pair of specific PCR primers was designed and applied. Approximately, 760 bp DNA fragment was amplified in the eight strains (Figure 2). Several genotypic methods applied various PCR protocols; using degenerate primers to detect and amplify the PHA synthase gene(s) have been reported (Solaiman et al., 2000; Sheu et al., 2001; Shamala et al., 2003; Solaiman and Ashby, 2005; Chien et al., 2007; Berekaa and Al Thawadi, 2012). Interestingly, successful application of polyphasic approach using combination of phenotypic and genotypic screening method was recorded by Chien et al. (2007). This polyphasic approach helps to overcome drawbacks of the individual detection method.

PHB accumulation by *Bacillus* sp. SW1-2

Among the positive strains a bacterium, previously isolated and identified as *B. megaterium* SW1-2 (Berekaa and Al Thawadi, 2012), was used for chemical and molecular analysis of PHB production. PHB was closely monitored during growth of *Bacillus megaterium* SW1-2 on modified E2 medium supplemented with glucose as a sole carbon source and in presence of beef extract. Results in figure 3 showed the growth and PHB accumulation

measured during a period of 96 hr. Obviously, maximum PHB production was attained (36% CDW) after 48 h. Amount of PHB was clearly decreased (15% CDW) at the end of incubation period. Similarly, *B. megaterium* OU303A, *B. cereus* SPV and other bacilli produces PHA co-polymer composed mainly of PHB during growth on modified E2 medium supplemented with glucose as a carbon source and in presence of traces of yeast extract instead of beef extract (Gouda et al., 2001; Law et al., 2001; Valappil et al., 2007b; Adwitiya et al., 2009; Reddy and Mahmood, 2009; Berekaa and Al Thawadi, 2012), support the results presented in this work. Interestingly, monitoring of PHB production in *B. megaterium* cells indicated that it is produced during logarithmic phase and maximally accumulated just prior to the formation of spores and was degraded during the process of sporulation (data not shown).

Chemical analysis of the polymer

Preliminary analysis of polymeric material digested with concentrated H_2SO_4 and scanned with UV-Vis spectrophotometer revealed a sharp peak at 235 nm characteristic of crotonic acid indicating the presence of PHB biopolymer (data not shown).

Characterization of the extracted PHB

C^{13} NMR analysis

Furthermore, C^{13}NMR analysis was used to determine the structure of the isolated polymer from *B. megaterium* SW1-2 grown on the modified E2 medium (Figure 4a). Four narrow lines appeared which were identical to the C^{13}NMR spectra of PHB, as reported previously (Doi and Abe, 1990). These four peaks were assignable to the methyl (CH3; 21.2 ppm), methylene (CH2; 42.7 ppm), methine (CH; 68.5 ppm) and carbonyl (C=O; 169.7 ppm) carbon resonance of PHB (Doi and Abe, 1990). This analysis thus confirmed the molecular composition of the polymer to be PHB.

H^1 NMR analysis

The extracted polymer was dissolved in 1 ml $CDCl_3$ followed by H^1NMR analysis. Three groups of signals characteristic of polymer PHB were seen in the spectrum (Figure 4b). A doublet at 1.26 ppm represented the methyl group (CH_3) coupled to one proton while a doublet of quadruplet at 2.45, 2.58 ppm resulted from methylene group (CH_2) adjacent to an asymmetric carbon atom bearing a single proton. The third signal was a multiplet at 5.2 ppm, which was attributed to a methyne group (CH). From the contribution of various groups to the NMR spectra, it was concluded that the biodegradable polymer accumulated in the bacterial biomass is in the form of PHB.

Figure 1. Alignment of nucleotide sequence of polyhydroxyalkanoate *pha*C gene from *B. megaterium* SW1-2 as compaired to other sequences of *pha*C synthase gene from different bacilli.

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B. cereus DC4    CGTGGTTTTGATGTATATAT GCTTGATTGGGGCACATTTG GTTTAGAAGATAGTCATTTG
B. cereus DC3    CGTGGTTTTGATGTATATAT GCTTGATTGGGGCACATTTG GTTTAGAAGATAGTCATTTG
B. cereus DC2    CGTGGTTTTGATGTATATAT GCTTGATTGGGGCACATTTG GTTTAGAAGATAGTCATTTG
B. sp INT005     CGTGGTTTTGATGTGTATAT GCTTGATTGGGGCACATTTG GTTTAGAAGATAGTCATTTG
B. sp CFR13      CGTGGTTTTGATGTGTATAT GCTTGATTGGGGCACATTTG GTTTAGAAGATAGTCATTTG
B. mega 1        CGTGGTTTTGACGTGTATTT GCTTGACTGGGGAACTCCAG GGCTTGAAGACAGCAATATG
B. mega 2        CGCGGTTTTGACGTGTATTT GCTTGACTGGGGAACTCCTG GGCTTGAAGACAGCAATATG
B. mega 3        CGCGGTTTTGACGTGTATTT GCTTGACTGGGGAACTCCTG GGCTTGAAGACAGCAATATG
B. meg SW1-2             GATGTGTATTT GCTTGACTGGGGAACTCCAG GGCTTGAAGACAGCAATATG
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                                    Forward
B. cereus DC4    AAATTTGATGATTTCGTGTT TGATTATATTGCAAAAGCAG TAAAAAAAGTAATGCGAACT
B. cereus DC3    AAATTTGATGATTTCGTGTT TGATTATATTGCAAAAGCAG TAAAAAAAGTAATGCGAACT
B. cereus DC2    AAATTTGATGATTTCGTGTT TGATTATATTGCAAAAGCAG TAAAAAAAGTAATGCGAACT
B. sp INT005     AAATTTGATGATTTTGTGTT TGATTATATTGCAAAAGCAG TGAAAAAAGTAATGCGAACT
B. sp CFR13      AAATTTGATGATTTTGTGTT TGATTATATTGCAAAAGCAG TGAAAAAAGTAATGCGAACT
B. mega 1        AAGCTAGATGATTATATTGT AGATTATATTCCAAAAGCGG CGAAAAAGGTGCTGCGCACT
B. mega 2        AAGCTAGATGATTATATTGT AGATTATATTCCAAAAGCGG CGAAAAAGGTGCTGCGCACT
B. mega 3        AAGCTAGATGATTATATTGT AGATTATATTCCAAAAGCGG CGAAAAAGGTGCTGCGCACT
B. meg SW1-2     AAGCTAGATGATTATATTGT AGATTATATTCCAAAAGCGG CGAAAAAGGTGCTGCGCACT
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B. cereus DC4    GCAAAATCGGACGAGATTTC TTTACTTGGTTATTGCATGG GTGGAACGCTAACTTCTATT
B. cereus DC3    GCAAAATCGGACGAGATTTC TTTACTTGGTTATTGCATGG GTGGAACGCTAACTTCTATT
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B. cereus DC2    GCAAAATCGGACGAGATTTC TTTACTTGGTTATTGTATGG GTGGAACGCTAACTTCTATT
B. sp INT005     GCAAAATCGGACGAGATTTC TTTACTTGGTTATTGCATGG GGGGAACGCTAACTTCTATT
B. sp CFR13      GCAAAATCGGACGAGATTTC TTTACTTGGTTATTGCATGG GGGGAACGCTAACTTCTATT
B. mega 1        TCTAAATCTCCTGATTTGTC TGTTCTTGGTTACTGCATGG GCGGAACTATGACATCTATT
B. mega 2        TCTAAATCTCCTGATTTGTC TGTTCTTGGTTACTGCATGG GCGGAACTATGACATCTATT
B. mega 3        TCTAAATCTCCTGATTTGTC TGTTCTTGGTTACTGCATGG GCGGAACCATGACATCTATT
B. meg SW1-2     TCTAAATCTCCTGATTTGTC TGTTCTTGGTTACTGCATGG GCGGAACCATGACATCTATT
                 *:***** . ** :* ** * *:******** ** **** * ***** .*.**:******
B. cereus DC4    TATGCAGCGCTTCATCCGCA CATGCCAATTCGTAATTTAA TTTTCATGACAAGTCCTTTT
B. cereus DC3    TATGCAGCGCTTCATCCGCA CATGCCAATTCGTAATTTAA TTTTCATGACAAGTCCTTTT
B. cereus DC2    TATGCAGCGCTTCATCCGCA CATGCCAATTCGTAATTTAA TTTTCATGACAAGTCCTTTT
B. sp INT005     TATGCGGCACTTCATCCGCA CATGCCAATTCGTAACCTAA TCTTTATGACAAGTCCTTTT
B. sp CFR13      TATGCGGCACTTCATCCACA TATGCCAATTCGTAACCTAA TCTTTATGACAAGTCCTTTT
B. mega 1        TTTGCTGCATTAAATGAAGA CTTGCCGATTAAAAACTTAA TTTTTATGACAAGTCCATTT
B. mega 2        TTTGCTGCATTAAATGAAGA CTTGCCGATTAAAAACTTAA TTTTTATGACAAGTCCATTT
B. mega 3        TTTGCTGCATTAAATGAAGA CTTGCCGATTAAAAACTTAA TTTTTATGACAAGTCCATTT
B. meg SW1-2     TTTGCTGCATTAAATGAAGA CTTGCCGATTAAAAACTTAA TTTTTATGACAAGTCCATTT
                 *:*** **. *:.** .. *  :****.***..:**  *** * ** ***********:***
B. cereus DC4    GATTTCTCTGAAACAGGATT GTATGGTCCTTTATTAGATG AGAAATACTTCAATTTAGAT
B. cereus DC3    GATTTCTCTGAAACAGGATT GTATGGTCCTTTATTAGATG AGAAATACTTCAATTTAGAT
B. cereus DC2    GATTTCTCTGAAACAGGATT GTATGGTCCTTTATTAGATG AGAAATACTTCAATTTAGAT
B. sp INT005     GATTTCTCTGAAACAGGATT ATATGGTCCTTTATTAGATG AGAAATACTTCAATCTAGAT
B. sp CFR13      GATTTCTCTGAAACAGGATT ATATGGTCCTTTATTAGATG AGAAATACTTCAATCTAGAT
B. mega 1        GATTTTTCGGATACAGGTTT ATACGGAGCATTCCTAGACG ATCGCTACTTTAATTTAGAT
B. mega 2        GATTTTTCGGATACAGGTTT ATACGGAGCATTCCTAGATG ATCGCTACTTTAATTTAGAT
B. mega 3        GATTTTTCGGATACAGGTTT ATACGGAGCATTCCTAGACG ATCGCTACTTTAATTTAGAT
B. meg SW1-2     GATTTTTCGGATACAGGTTT ATACGGAGCATTCCTAGACG ATCGCTACTTTAATTTAGAT
                 ***** ** **:*****:** .** **: *:**. **** * * ...***** *** *****
B. cereus DC4    AAAGCGGTTGATACATTTGG AAATATTCCGCCAGAAATGA TTGATTTCGGAAACAAAGTG
B. cereus DC3    AAAGCGGTTGATACATTTGG AAATATTCCGCCAGAAATGA TTGATTTCGGAAACAAAATG
B. cereus DC2    AAAGCGGTTGATACATTTGG AAATATTCCGCCAGAAATGA TTGATTTCGGAAACAAAATG
B. sp INT005     AAAGCGGTTGATACATTTGG AAATATTCCGCCAGAAATGA TTGATTTCGGAAACAAAATG
B. sp CFR13      AAAGCGGTTGATACATTTGG AAATATTCCGCCAGAAATGA TTGATTTCGGAAACAAAATG
B. mega 1        AAAGCAGTAGATACATTCGG AAACATCCCTCCAGAGATGA TTGACTTTGGAAACAAGATG
B. mega 2        AAAGCAGTAGATACATTCGG AAACATCCCTCCAGAGATGA TTGACTTTGGAAACAAGATG
B. mega 3        AAAGCAGTAGATACATTCGG AAACATCCCTCCAGAGATGA TTGACTTTGGAAACAAAATG
B. meg SW1-2     AAAGCAGTAGATACATTCGG AAACATCCCTCCAGAGATGA TTGACTTTGGAAACAAGATG
                 *****.**:******** ** *** ** ** *****.**** **** ** ********..**
B. cereus DC4    TTAAAGCCAATTACGAACTT TGTTGGTCCATATGTTGCTC TAGTAGATCGTTCAGAGAAT
B. cereus DC3    TTAAAGCCAATTACGAACTT TGTTGGTCCATATGTTGCTC TAGTAGATCGTTCAGAGAAT
B. cereus DC2    TTAAAGCCAATTACGAACTT TGTTGGTCCATATGTTGCTC TAGTAGATCGTTCAGAGAAT
B. sp INT005     TTAAAACCAATTACGAACTT TGTTGGTCCATATGTTGCTT TAGTAGATCGTTCAGAGAAT
B. sp CFR13      TTAAAACCAATTACGAACTT TGTTGGTCCATATGTTGCTT TAGTAGATCGTTCAGAGAAT
B. mega 1        TTAAAGCCAATCACGAATTT CTATGGCCCATATGTAACGT TGGTGGACCGTTCGGAAAAT
B. mega 2        TTAAAGCCAATCACGAATTT CTACGGCCCGTATGTAACGT TGGTGGACCGTTCGGAAAAT
B. mega 3        TTAAAGCCAATCACGAATTT CTATGGCCCATATGTAACGC TAGTGGACCGTTCGGAAAAT
B. meg SW1-2     TTAAAGCCAATCACGAATTT CTATGGCCCATATGTAACGT TGGTGGACCGTTCGGAAAAT
                 *****.***** ***** **   : ** **.*****:.*   *.**.** *****.**.***
B. cereus DC4    GAACGCTTCGCCGAAAGCTG GAGATTGGTTCAAAAGTGGG TTGGTGATGGTATTCCGTTC
B. cereus DC3    GAACGCTTCGTCGAAAGCTG GAGATTGGTTCAAAAGTGGG TTGGTGATGGTATTCCGTTC
B. cereus DC2    GAACGCTTCGTCGAAAGCTG GAGATTGGTTCAAAAGTGGG TTGGTGATGGTATTCCGTTC
B. sp INT005     GAGCGCTTCGTTGAAAGCTG GAGGTTAGTTCAAAAGTGGG TTGGCGATGGCATTCCGTTC
B. sp CFR13      GAGCGCTTCGTTGAAAGCTG GAGGTTAGTTCAAAAGTGGG TTGGCGATGGCATTCCGTTC
B. mega 1        CAGCGGTTTGTTGAAAGCTG GAAGCTAATGCAAAAGTGGG TTGCTGACGGAATCCCATTT
B. mega 2        CAGCGGTTTGTTGAAAGCTG GAAGCTAATGCAAAAGTGGG TTGCTGACGGAATCCCATTT
B. mega 3        CAGCGGTTTGTTGAAAGCTG GAAGCTAATGCAAAAGTGGG TTGCTGACGGAATCCCATTT
B. meg SW1-2     CAGCGGTTTGTTGAAAGCTG GAAGCTAATGCAAAAGTGGG TTGCTGACGGAATCCCATTT
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                 *.** ** *  ******** **.. *..* ********** ***  ** ** ** **.**
B. cereus DC4    CCAGGTGAATCATACAGACA GTGGATTCGTGATTTTTATC AAAATAATAGATTGGTTAAG
B. cereus DC3    CCAGGTGAATCATACAGACA GTGGATTTGTGATTTTTATC AAAATAATAAATTGGTTAAG
B. cereus DC2    CCAGGTGAATCATACAGACA GTGGATTCGTGATTTTTATC AAAATAATAAATTGGTTAAG
B. sp INT005     CCAGGTGAATCATACAGACA GTGGATTCGTGATTTTTATC AAAATAATAAATTGGTTAAG
B. sp CFR13      CCAGGTGAATCATACAGACA GTGGATTCGTGATTTTTATC AAAACAATAAATTGGTTAAG
B. mega 1        GCTGGTGAAGCTTATCGTCA GTGGATTCGTGACTTCTATC AACAAAATAAATTAATCAAT
B. mega 2        GCTGGCGAAGCTTATCGTCA GTGGATTCGTGACTTCTATC AACAAAACAAACTAATCAAT
B. mega 3        GCTGGTGAAGCTTATCGTCA GTGGATTCGTGACTTCTATC AACAAAATAAATTAATCAAT
B. meg SW1-2     GCTGGTGAAGCTTATCGTCA GTGGATTCGTGACTTCTATC AACAAAATAAATTAATCAAT
                 *:** *** *:** .*:** ******* **** ** **** **.* ** *.* *..* **
B. cereus DC4    GGTGAACTCGTTATTCGCGG ACAAAAGGTAGATCTTGCAA ATATTAAGGCGAATGTCTTA
B. cereus DC3    GGTGAACTCGTTATTCGCGG ACAAAAGGTAGATCTTGCAA ATATTAAGGCGAATGTCTTA
B. cereus DC2    GGTGAACTCGTTATTCGCGG ACAAAAGGTAGATCTTGCAA ATATTAAGGCGAATGTCTTA
B. sp INT005     GGTGAACTCGTTATTCGCGG ACAAAAGGTAGACCTTGCAA ATATTAAGGCGAATGTCTTA
B. sp CFR13      GGTGAACTCGTTATTCGCGG ACAAAAGGTAGACCTTGCAA ATATTAAGGCGAATGTCTTA
B. mega 1        GGTGAACTTGAAGTTCGCGG ACGCAAAGTAGATTTGAAAA ATATTAAAGCTAATATTTTA
B. mega 2        GGTGAACTTGAAGTTCGCGG ACGCAAAGTAGATTTAAAAA ATATTAAAGCTAATATTTTA
B. mega 3        GGTGAACTTGAAGTTCGCGG ACGCAAAGTAGATTTGAAAA ATATTAAAGCTAACATTTTA
B. meg SW1-2     GGTGAACTTGAAGTTCGCGG ACGCAAAGTAGATTTGAAAA ATATTAAAGCTAATATTTTA
                 ******** *::.******* **..**.*****  * ..** *******.** ** .* ***
B. cereus DC4    AATATTTCCGGGAAACGTGA TCATATCGCTTTGCCATGTC AAGTAGAAGCATTACTAGAC
B. cereus DC3    AATATTTCCGGGAAACGTGA TCATATCGCTTTGCCATGTC AAGTAGAAGCATTACTAGAC
B. cereus DC2    AATATTTCCGGGAAACGTGA TCATATCGCTTTGCCATGTC AAGTAGAAGCATTACTAGAC
B. sp INT005     AATATTTCAGGGAAACGTGA TCATATCGCCCTGCCGTGCC AAGTAGAAGCGTTGCTAGAT
B. sp CFR13      AATATTTCAGGGAAACGTGA TCATATCGCCCTGCCATGCC AAGTAGAAGCGTTGCTAGAT
B. mega 1        AACATTGCTGCTAGCCGTGA TCATATTGCGATGCCGCATC AAGTGGCAGCTTTAATGGAC
B. mega 2        AACATTGCTGCTAGCCGTGA TCATATTGCGATGCCGCATC AAGTGGCAGCTTTAATGGAC
B. mega 3        AACATTGCTGCTAGCCGTGA TCATATTGCGATGCCACATC AAGTGGCAGCATTAATGGAC
B. meg SW1-2     AACATTGCTGCTAGCCGTGA TCATATTGCGATGCCGCATC AAGTGGCAGCTTTAATGGAC
                 ** *** * *  *..***** ****** **  ****. . * ****.*.*** **..*.**
B. cereus DC4    CATATCTCTAGCACAGATAA ACAATATGTATGTTTACCAA CAGGGCATATGTCTATCGTT
B. cereus DC3    CATATTTCTAGCACAGATAA ACAATATGTATGTTTACCAA CAGGGCATATGTCTATCGTT
B. cereus DC2    CATATTTCTAGCACAGATAA ACAATATGTATGTTTACCGA CAGGGCATATGTCCATCGTT
B. sp INT005     CATATTTCTAGCACAGATAA ACAATATGTATGTTTACCAA CGGGACATATGTCGATTGTT
B. sp CFR13      CATATTTCTAGCACAGATAA ACAATATGTATGTTTACCAA CGGGACATATGTCGATTGTT
B. mega 1        GCTGTTTCAAGTGAAGATAA AGAGTACAAATTGTTGCAAA CAGGTCACGTATCTGTTGTA
B. mega 2        GCTGTTTCAAGTGAAGATAA AGAGTATAAATTGTTGCAAA CAGGTCACGTATCTGTTGTA
B. mega 3        GCTGTTTCAAGTGAAGATAA AGAGTATAAATTGTTGCAAA CAGGTCACGTATCTGTTGTA
B. meg SW1-2     GCTGTTTCAAGTGAAGATAA AGAGTATAAATTGTTGCAAA CAGGTCACGTATCTGTTGTA
                  .*.* **:** ..****** * *.** .:**  **.*..* *.** ** .*.** .* **:
B. cereus DC4  TACGGTGGAACAGCTGTAAA ACAAACATATCCGACGATTG GAAATTGGCTTGAAGAGCGT
B. cereus DC3    TACGGTGGAACAGCTGTAAA ACAAACATATCCGACGATTG GAAATTGGCTTGAAGAGCGT
B. cereus DC2    TACGGTGGAACAGCTGTAAA ACAAACATATCCGACGATTG GAAATTGGCTTGAAGAGCGT
B. sp INT005     TACGGTGGAACAGCGGTAAA ACAAACGTATCCGACGATTG GAGACTGGCTTGACGAGCGC
B. sp CFR13      TACGGTGGAACAGCGGTAAA ACAAACGTATCCGACGATTG GAGACTGGCTTGAAGAGCGT
B. mega 1        TTTGGTCCAAAAGCAGTGAA GGAAACATATCCTTCAATCG GCGATTGGCTAGAAAAACGC
B. mega 2        TTTGGTCCAAAAGCAGTGAA GGAAACATATCCTTCAATCG GCGATTGGCTAGAAAAACGC
B. mega 3        TTTGGTCCAAAAGCAGTGAA GGAAACATATCCTTCAATCG GCGATTGGCTAGAAAAACGC
B. meg SW1-2     TTTGGTCCAAAAGCAGTGAA GGAAACATATCCTTCAATCG GCGATTGGCT
                 *: ***  **.*** **.** . ****.***** :*.** * *..* *****:**..*.**
                                                    Reverse
```

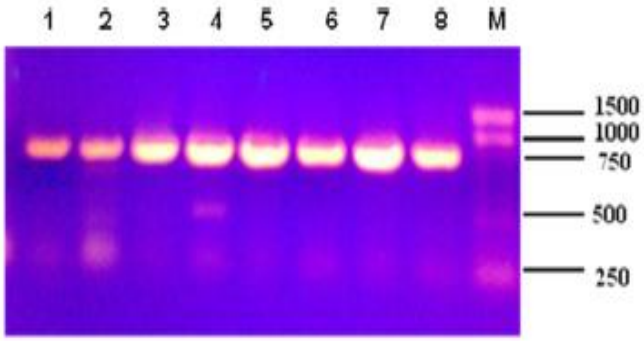

Figure 2. Agarose gel electrophoresis of PCR products from different bacilli (Lanes 1-6,8) and *B. megaterium* SW1-2 (Lane 7) with gene specific primer set and 1.0 kbp DNA ladder (lanes 9).

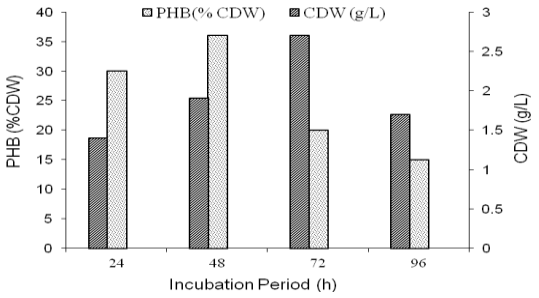

Figure 3. Growth and polyhydroxybutyrate (PHB) production by *B. megaterium* WS1-2 during different time intervals.

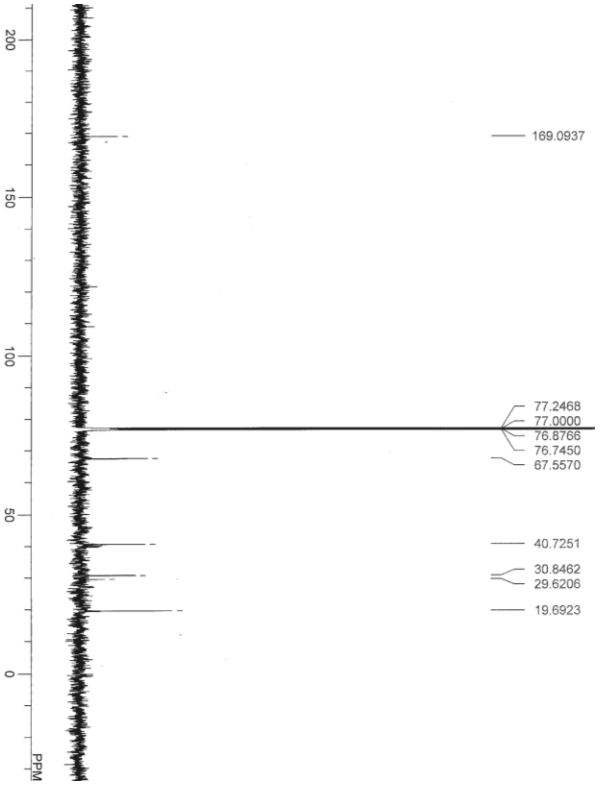

Figure 4a. C^{13}-NMR Spectroscopy of PHB biopolymer produced from *B. megaterium* SW1-2.

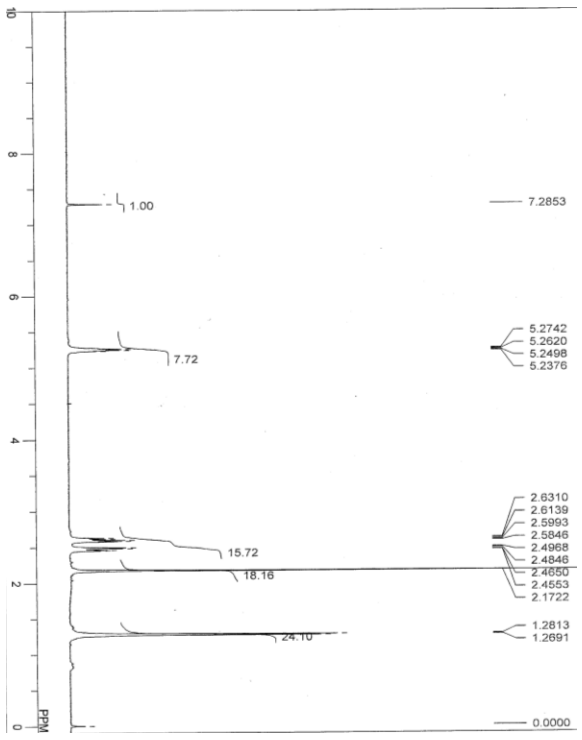

Figure 4b. H^{1}-NMR Spectroscopy of PHB biopolymer produced from *B. megaterium* SW1-2.

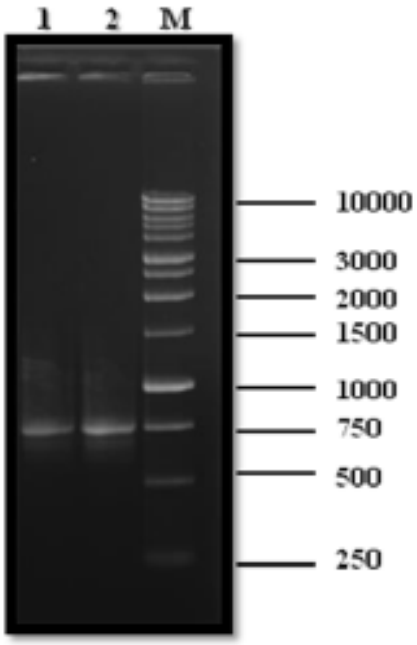

Figure 5. Agarose gel electrophoresis (1%) of PCR products from *B. megaterium* SW1-2 (Lanes 1-2) with gene specific primers set and 1.0 kbp DNA ladder (Lane M).

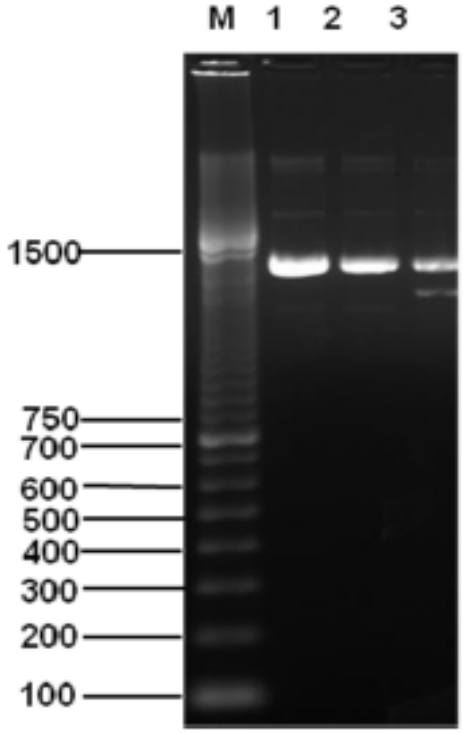

Figure 6. Agarose gel electrophoresis (1%) of plasmid minipreps from *B. megaterium* SW1-2 cloned PCR product onto pGEM-T-Easy vector (Lane 1-3) and 100 bp DNA ladder (Lane M).

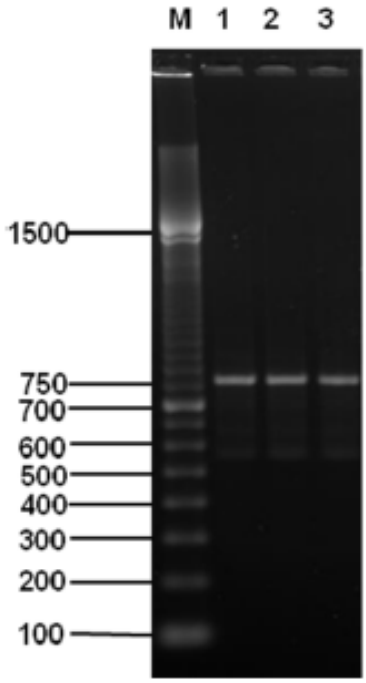

Figure 7. Agarose gel electrophoresis (1%) of PCR fragment from *B. megaterium* SW1-2 (Lane 1-3) amplified with T7 and SP6 primer set and 100 bp DNA ladder (Lane M).

Analysis of *B. megaterium* SW1-2 *pha*C synthase gene

The PCR fragment amplified from genomic DNA of *B. megaterium* SW1-2 was cloned in pGEM-T-Easy vector, transform into *Escherichia coli* DH5a competent cells. Recombinant *E. coli* DH5a harboring the pGEM-T-Easy vector was screened in selective LB/IPTG/X-gal/Ampicillin/agar plates. Plasmids prepared from some positive clones, isolated and purified (Figure 6). The presence of a cloned DNA insert was tested and verified by PCR amplification of the plasmid with T7/SP6 primers, which anneal to the vector region flanking the DNA insert (Figure 7). Several clones containing the DNA insert were then sequenced to ensure their uniqueness. Sequencing revealed that most of the amplified products were uniform and corresponded to *pha*C synthase of *Bacillus* sp. Nucleotide sequences corresponding to 761 bp compared with the nucleotide sequences deposited in the GenBank database using the Blast family program (Figure 1). The sequence was deposited in GenBank under accession number JQ755810. Indeed, the sequence of *pha*C synthase gene of *B. megaterium* revealed very close identity to other *pha*C gene sequences from other bacilli. It showed 99% identity to *pha*C gene for polyhydroxyalkanoate synthase of *Bacillus megaterium* DSM319, *Bacillus megaterium* QM

B1551, and 98% to synthase of *Bacillus megaterium* WSH-002 (Liu et al., 2011). However, the sequence showed 73% identity to synthase of *Bacillus mycoides* strain DFC1 and the *Bacillus thuringiensis* serovar finitimus YBT-020 (Zhu et al., 2011) and 72% to synthase of *Bacillus* sp. INT005 (Satoh et al., 2002). In concurrence, successful application of different molecular approaches for detection of PHAs accumulation by a variety of bacteria was reported by many scientists (Sheu et al., 2000; Castano et al., 2007; Chien et al., 2007).

Conclusion

PHAs are synthesized and intracellular accumulated as granules in many bacteria. Results revealed that phenotypic detection of PHAs accumulating bacteria can be supported by genotypic detection method. This polyphasic approach satisfies the minimal industrial standards for screening potentially useful organisms for PHA production. Pair of specific conserved primers (P1 and P2) can be used as universal primers to amplify partial segment from *pha*C gene of several bacilli among of them *B. megaterium* SW1-2. Indeed, the ability to produce PHB was confirmed by C^{13} and H^{1}NMR spectroscopy. Furthermore, *pha*C synthase gene of *B. megaterium* SW1-2 showed 98%-99% identity to *pha*C gene for polyhydroxyalkanoate synthase of many *Bacillus megaterium* strains. However, the sequence showed 73% identity to synthase of *Bacillus mycoides* strain DFC1 and the *Bacillus thuringiensis* serovar finitimus YBT-020 and 72% to synthase of *Bacillus* sp. INT005. Consequently, cloning of the whole synthase gene might allow for recombinant production of the biodegradable biopolymer to be used in the field of medical and environmental biotechnology.

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